Determination of Mitochondrial Function and Site-Specific Defects in Electron Transport in Duodenal Mitochondria in Broilers with Low and High Feed Efficiency

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ABSTRACT Duodenal mitochondria were isolated from broiler breeder males with high (0.79 ± 0.01, n = 9) and low (0.63 ± 0.02, n = 9) feed efficiency (FE) to assess relationships of FE with duodenal mitochondrial function and site-specific defects in electron transport. Sequential additions of adenosine diphosphate (ADP) resulted in 1) higher respiratory control ratio (RCR; an index of respiratory chain coupling) in high FE mitochondria provided succinate, and 2) higher ADP to oxygen ratio (ADP:O; an index of oxidative phosphorylation) in low FE mitochondria provided NADH-linked substrates (malate, pyruvate, or both). Basal electron leak, measured as H2O2 production, was greater in low FE mitochondria provided succinate ($P = 0.08$) or NADH-linked substrates. As H2O2 levels were elevated in low FE compared with high FE mitochondria by complex I ($P \leq 0.07$) and complex II inhibition, the higher basal electron leak in low FE mitochondria was apparently due to site-specific defects in electron transport at complexes I and II. Elevations in H2O2 above basal levels indicated that high FE mitochondria may also exhibit transport defects at complexes I and III. Despite an ability to produce adenosine triphosphate (ATP) that was equal or superior to that demonstrated in high FE duodenal mitochondria, low FE mitochondria exhibited a greater inherent degree of electron leak. The results provide insight into the role that duodenal mitochondria play in the phenotypic expression of FE in broilers.

(Key words: broiler, duodenum mitochondria, electron leak, feed efficiency, respiratory control ratio)

INTRODUCTION

As feed represents about 50 to 70% of the cost of broiler production, feed efficiency (FE, gain to feed) or feed conversion ratio (FCR, feed to gain) remains one of the most important traits in commercial animal breeding programs. Genetic selection for increased broiler performance has resulted in a 250 to 300% improvement in body weight and FE in 1991 and 2001 broiler strains compared with a 1957 random bred control population (Havenstein et al., 1994, 2003; Chapman et al., 2003). Availability of feed additives that support optimum growth and advances in broiler production such as feed formulation that allow close approximation of the nutrient requirements have also improved FE. However, despite these advances, as much as 10% variation in growth and FE still exists within broiler lines (Emmerson, 1997).

Recent evidence suggests that mitochondrial function or biochemistry may be associated with FE in broilers (Bottje et al., 2002; Iqbal et al., 2004) and rats (Lutz and Stahly, 2003). Whereas various studies have reported differences in mitochondrial oxygen utilization with different breeds of animals (e.g., Mukherjee et al., 1970; Dziewiecki and Kolataj, 1976; Brown et al., 1986) or dietary manipulations (e.g., Renner et al., 1979; Toyomizu et al., 1992a,b), to our knowledge, the reports of Bottje et al. (2002) and Lutz and Stahly (2003) are the only studies that have suggested a direct relationship between mitochondrial function and FE that have excluded breed and dietary effects.

Mitochondria are organelles found in all eukaryotic cells whose major function is to generate cellular energy (adenosine triphosphate; ATP). Approximately 90% of the total ATP production from the complete oxidation of glucose to carbon dioxide and water is generated by

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Abbreviation Key: ADP = adenosine diphosphate; ADP:O = adenosine diphosphate to oxygen ratio; ATP = adenosine triphosphate; DCFH-DA = 2′,7′-dichlorofluorescin diacetate; EGTA = ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid; FE = feed efficiency; HEPES = N-[2-hydroxyethylpiperazin]-N′-[2-ethanesulfonic acid]; $O_2^-$ = superoxide; RCR = respiratory control ratio; ROS = reactive oxygen species; TTFA = thenoyltrifluoroacetone.
mitochondria via oxidative phosphorylation (Lehninger et al., 1993). The inner mitochondrial membrane bears the components of the electron transport chain, also known as the respiratory chain or oxidative phosphorylation system. The respiratory chain consists of 5 multiprotein enzyme complexes, complexes I, II, III, IV, and V [ATPase; coenzyme Q (CoQ) and cytochrome c (cyt c), and 2 mobile electron carriers, substrate donate electrons at complexes I and II, respectively. Electrons (e⁻) are transferred to O₂, the terminal electron acceptor, which is subsequently reduced to water. Electron flow through complexes I, III, and IV is accompanied by proton transfer from the mitochondrial matrix to the inner membrane space creating a proton-motive force, which drives protons back into the matrix through ATP synthase and provides the energy for ATP synthesis.

Mitochondrial dysfunction such as increased ROS production has been linked to the pathogenesis of many human diseases, including but not limited to, diabetes, Alzheimer’s, Parkinson’s, ischemic reperfusion injury, and in aging (e.g., Fiegal and Shapiro, 1979; Shigenaga et al., 1994; Benzi and Moretti, 1995; Herrero and Barja, 1998; Madesh et al., 2000; Rustin and Rotig, 2001). In broilers, mitochondrial dysfunction has been implicated to pulmonary hypertension syndrome (Cawthon et al., 1999, 2001; Tang et al., 2000, 2002; Iqbal et al., 2001). Bottje et al. (2002) also reported that muscle mitochondrial dysfunction including increased ROS production may contribute to the phenotypic expression of low FE in broilers. As the small intestine is the major site of nutrient absorption requiring considerable amounts of ATP, the purpose of this study was to extend our findings from those of the muscle by evaluating duodenal mitochondrial function in broilers from the same genetic line and fed the same diet. Our hypothesis is that intestinal mitochondrial function could also be important in the phenotypic expression of FE in broilers. Major objectives of this study were: 1) to evaluate relationships between duodenal mitochondrial function (respiratory chain coupling and oxidative phosphorylation) and FE and 2) to assess sites and amounts of electron leak in duodenum mitochondria by measuring H₂O₂ production in low and high FE broilers.

**MATERIALS AND METHODS**

**Birds and Management**

In this study, eighteen 7-wk-old birds that exhibited the highest or lowest FE were identified within a group of 100 breeder male replacement stock3 tested for FE from 6 to 7 wk of age. The birds were color-coded such that we were not aware which color belonged to the high and low FE groups until completion of the experiments. Birds were transported to the University of Arkansas, where they were housed individually in similar cages (51 × 51 × 61 cm) and environmental conditions (25°C, 15L:9D) and were fed the same diet provided during the FE trial (20.5% protein, 3,280 kcal/kg). Birds were provided free access to feed and water. After a 5-d acclimation, one bird per day was randomly selected from each group for isolation of duodenal mitochondria, with group selection alternated on each day. Each bird was euthanized with an overdose of sodium pentobarbital by intravenous injection into the wing vein. Data were also obtained for weight, length, and diameter of the duodenal loop and mucosa weight.

**Duodenal Mitochondria Isolation**

Mitochondria were isolated by standard differential centrifugation as described by Lawrence and Davies...
TABLE 1. Six- to 7-wk growth performance of broilers with low or high feed efficiency (FE) \(^1\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>High FE ((n = 9))</th>
<th>Low FE ((n = 9))</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-wk BW, g</td>
<td>2,331 ± 28</td>
<td>2,310 ± 21</td>
<td>0.544</td>
</tr>
<tr>
<td>7-wk BW, g</td>
<td>3,283 ± 39</td>
<td>3,065 ± 48</td>
<td>0.003</td>
</tr>
<tr>
<td>BW gain, g</td>
<td>951 ± 30</td>
<td>756 ± 36</td>
<td>0.001</td>
</tr>
<tr>
<td>Feed intake, g</td>
<td>1,205 ± 30</td>
<td>1,190 ± 47</td>
<td>0.790</td>
</tr>
<tr>
<td>FE, g of gain/g of feed</td>
<td>0.79 ± 0.01</td>
<td>0.63 ± 0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FCR, (^2) g of feed/g of gain</td>
<td>1.27 ± 0.01</td>
<td>1.58 ± 0.02</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^1\) For details see Materials and Methods. Values are mean ± SEM of \(n\) shown in parentheses.

\(^2\) Feed conversion ratio.

TABLE 2. Duodenal loop (DL) physical characteristics of broilers with low or high feed efficiency (FE) \(^1\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>High FE ((n = 7))</th>
<th>Low FE ((n = 8))</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL weight, g</td>
<td>43 ± 4</td>
<td>46 ± 4</td>
<td>0.61</td>
</tr>
<tr>
<td>DL length, cm</td>
<td>46 ± 2</td>
<td>49 ± 2</td>
<td>0.42</td>
</tr>
<tr>
<td>DL diameter, cm</td>
<td>0.72 ± 0.04</td>
<td>0.73 ± 0.03</td>
<td>0.89</td>
</tr>
<tr>
<td>DL area, cm(^2)</td>
<td>53 ± 3</td>
<td>56 ± 2</td>
<td>0.38</td>
</tr>
<tr>
<td>Mucosa weight, g</td>
<td>11 ± 1</td>
<td>10 ± 1</td>
<td>0.61</td>
</tr>
<tr>
<td>Mucosa/DL area, g/cm(^2)</td>
<td>0.21 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>0.30</td>
</tr>
<tr>
<td>Mitochondrial protein, mg/mL</td>
<td>2.1 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>0.46</td>
</tr>
</tbody>
</table>

\(^1\) For details see Materials and Methods. Values are means ± SEM of \(n\) shown in parentheses.

(1986) with modifications. Briefly, the duodenal loop was quickly excised from the anesthetized bird and immediately placed in a beaker containing ice-cold oxygenated solution A [160 mM sucrose, 110 mM mannitol, 2 mM HEPES, 11 mM Tris-HCl, 0.25 mM ethylene glycol-bis (\(\beta\)-aminoethylether)-N,N,N',N' tetraacetic acid (EGTA), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4]. The pancreas was carefully removed, and the duodenal loop was cut in half. The luminal side was flushed with solution A, and the remaining digesta was washed with the same solution and then wiped gently with Whatman #2 filter paper to blot solution A and wipe off mucin. The mucosa was separated from the musculature by scraping with a microscope slide.

Approximately 10 g of mucosa was mixed with DEAE-cellulose suspension [8 g of DEAE-cellulose in 80 mL of isolation medium A (70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 0.5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.37 g fatty acid-free BSA/100 mL, pH 7.4), 175 U/mg heparin, and 1 mM dithiothreitol]. After 2 min, 50 mL of isolation medium A was added to the suspension, and the mucosal cells were disrupted by gentle homogenization with 6 to 7 strokes in a Potter-Elvehjem vessel with a Teflon pestle. The homogenate was diluted by a further addition of 150 mL of isolation medium A and centrifuged at 750 \(\times\) g for 10 min. The supernatant containing mitochondria was filtered through nylon cloth \(^4\) and centrifuged at 9,800 \(\times\) g for 7 min. The mucus layer from the mitochondrial pellets was removed. The mitochondria were then resuspended in 35 mL of isolation medium A and centrifuged again at 12,100 \(\times\) g for 7 min. The mitochondrial pellet was further enriched by subjecting it to a final spin (12,100 \(\times\) g for 7 min) in 35 mL of isolation medium B (70 mM sucrose, 220 mM mannitol, 2 mM HEPES, and 1.2 g of fatty acid-free BSA/100 mL, pH 7.4). The final mitochondrial pellet was suspended in isolation medium B and stored on ice for functional assays. All procedures were carried out at 4°C.

Mitochondrial protein was determined with a Lowry assay,\(^5\) with BSA as a standard, according to Lawrence and Davies (1986). The purity of isolated mitochondria was evaluated for each mitochondrial preparation by measuring the activity of citrate synthase (mitochondrial marker) according to Srere (1969), with modifications. Citrate synthase activities (U/mg of protein) were 257 ± 23 and 265 ± 20 for high and low FE mitochondria, respectively. As mitochondrial protein and values of citrate synthase activity were not different between the high and low FE birds, the purity of mitochondrial preparation was considered to be similar between groups.

Assessment of Mitochondrial Function

Duodenal mitochondrial function was assessed by monitoring oxygen consumption polarographically according to Estabrook (1967). Oxygen consumption rate (expressed as nmol of monomeric oxygen/min per milligram of protein) was measured with a Clark-type oxygen electrode\(^6\) equipped with a magnetic stirrer and thermostatically controlled respiration chamber set at 37°C. Equal amounts (based on protein concentration) of freshly isolated mitochondria were added to the respiration chamber containing 900 \(\mu\)L of RCR reaction buffer (220 mM Tris-mannitol, 70 mM sucrose, 2 mM HEPES, 2.5 mM KH₂PO₄, 2.5 mM MgCl₂, 0.75 mM EDTA, 0.5 mM, and 0.13 g BSA/100 mL, pH 7.4) and 10 mM each of succinate (an FADH₂-linked energy substrate), pyruvate, or malate or 10 mM malate and 2.5 mM pyruvate (NADH-linked energy substrates).

In the presence of respiration buffer and an energy substrate, isolated mitochondria exhibit an initial slow rate of oxygen uptake (state 2). Upon addition of adenine diphosphate (ADP), an immediate increase in oxygen utilization is observed as ADP is converted to ATP (state 3). As mitochondrial protein and values of citrate synthase activity were not different between the high and low FE birds, the purity of mitochondrial preparation was considered to be similar between groups.

\(^4\) Performix 900, Berkshire Corporation, Great Barrington, MA.

\(^5\) Kit 610-A, Sigma Chemical Co., St. Louis, MO.

\(^6\) Yellow Springs Instruments Co., Yellow Springs, OH.
Mitochondrial \( \text{H}_2\text{O}_2 \) Production and Site-Specific Defects in Electron Transport

Figure 2 represents the electron transport chain (ETC) showing sites of chemical inhibition used to determine sites and amounts of electron leak in this study. An increase in \( \text{H}_2\text{O}_2 \) generation following chemical inhibition indicates that the site of electron leakage is between the site of inhibition and entry of substrate into the electron transport chain (Barja, 1999). In the presence of FADH\(_2\) linked substrate, electron transfer from complex II to ubiquinone was inhibited with 4,4,4-trifluoro-1-[2-thienyl]-1,3-butanediol (TFFA). Malonate, a competitive inhibitor of succinate dehydrogenase, was also used to inhibit entry of electrons to complex II. With NADH-linked substrates, rotenone was used to block electron transfer from complex I to ubiquinone, and myxothiazol and antimycin A inhibited electron flow into center \( \alpha \) (toward the inner mitochondrial membrane) of complex III, respectively. If a site-specific defect exists in the electron transport at any of these sites of chemical inhibition, electrons will leak from the respiratory chain and consume oxygen by univalent reduction that results in the formation of superoxide (\( \text{O}_2^- \)). In the presence of superoxide dismutase, \( \text{O}_2^- \) is dismutated to \( \text{H}_2\text{O}_2 \) (Chance et al., 1979).

The mitochondrial production of \( \text{H}_2\text{O}_2 \) was measured using dichlorofluorescein diacetate (DCFH-DA)\(^7\) fluorescent probe as previously described (Bass et al., 1983; Iqbal et al., 2001). DCFH-DA is a nonpolar compound that can readily diffuse into cells, where it is hydrolyzed by esterases to the nonfluorescent dichlorofluorescein (DCFH). The DCFH is then oxidized by \( \text{H}_2\text{O}_2 \) to a fluorescent product, dichlorofluorescein, which fluoresces when exposed to ultraviolet light in the presence of \( \text{H}_2\text{O}_2 \) and peroxidase (Rota et al., 1999; Bilski et al., 2002). The increase in dichlorofluorescein fluorescence was found to be highly correlated with \( \text{H}_2\text{O}_2 \) concentrations (Iqbal et al., 2001). Briefly, 45 \( \mu\text{L} \) of mitochondrial sample (2 mg/mL) was added in a 96-well microtiter plate containing 48 \( \mu\text{L} \) of \( \text{H}_2\text{O}_2 \) buffer (145 mM KCl, 30 mM HEPES, 5 mM KH\(_2\)PO\(_4\), 3 mM MgCl\(_2\), and 0.1 mM EGTA; pH 7.4), 52 \( \mu\text{M} \) DCFH-DA, and 10 \( \mu\text{M} \) each of succinate, malate, or pyruvate as energy substrates. Superoxide dismutase (10 U/well) was added to each well to convert all \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) with values corrected for blanks and residual fluorescence by catalase (Iqbal et al., 2001). Final concentrations of inhibitors used were: 10 \( \mu\text{M} \) of rotenone; 13 \( \mu\text{M} \) each of antimycin A, myxothiazol and TFFA; and 7 \( \mu\text{M} \) of malonate. The reaction mixture was incubated at 37°C, and the change in fluorescence was recorded for 20 min with a FLX800 Microplate Fluorescence Reader\(^8\) set at a sensitivity of 110 and excitation/emission wavelengths of 485 and 528 nm, respectively. \( \text{H}_2\text{O}_2 \) values were calculated from a standard curve with known amounts of \( \text{H}_2\text{O}_2 \), and results were expressed as nanomoles of \( \text{H}_2\text{O}_2 \) per minute per milligram of mitochondrial protein.

**Statistical Analyses**

Figure 3 was analyzed with regression analysis and all other data were analyzed with one-way ANOVA using *JMP 5.0* statistical software.\(^9\) Means were separated by Student’s \( t \)-test, and data are presented as the mean ± SEM. With the \( \text{H}_2\text{O}_2 \) data, multiple comparisons were performed with the same mean and errors were calculated from the observed errors on the original values, and errors were also reported as the standard error of the means. A probability level of \( P \leq 0.05 \) was considered significant unless stated otherwise.

**RESULTS**

**Growth Performance and Duodenal Loop Data**

Initial BW (at 6 wk) was not different, but the high FE birds were heavier at 7 wk due to faster growth rate with
Mitochondrial Function

Values for respiration rates, expressed as natoms of oxygen per minute per milligram of protein are provided in Table 3. With succinate, an FADH2-linked substrate, respiration rates were not different after the first addition of ADP. A lower state 4 respiration rate was observed in high FE duodenum mitochondria following the second addition of ADP. When NADH-linked substrates (malate, malate-pyruvate, and pyruvate) were used as electron donors, no differences were observed in respiration rates between the 2 groups.

Mitochondrial H2O2 Production

In this paper, the terms electron leak and H2O2 production are used synonymously. Results of duodenal mitochondrial H2O2 production with FADH2 (succinate) and NADH (malate, pyruvate, and malate-pyruvate)-linked substrates are shown in Figure 4. Basal H2O2 production is represented in mitochondria that were provided with no inhibitor (NI). As shown in Figure 4A, basal H2O2 production was marginally greater ($P < 0.08$) in low than in high FE mitochondria provided succinate. Inhibition of...
observed that the magnitude of H$_2$O$_2$ production was 10-fold lower in mitochondria provided pyruvate (Figure 4C) and pyruvate (Figure 4D). With pyruvate as an energy substrate, H$_2$O$_2$ production was significantly lower than ADP.O values within each column ($P < 0.05$).

The basal level of H$_2$O$_2$ production in low FE mitochondria was greater than high FE values with malate (Figure 4B), malate-pyruvate (Figure 4C) and pyruvate (Figure 4D). When mitochondria oxidized malate, inhibition of electron transport at complex I with rotenone and at complex III with antimycin A and myxothiazol (center o) significantly increased H$_2$O$_2$ production in high FE mitochondria. Low FE mitochondrial H$_2$O$_2$ values with complexes I and III inhibition were also numerically increased compared with the basal values, but the increase in magnitude was not significant. When the 2 groups were compared within an inhibitor treatment, H$_2$O$_2$ values were higher ($P < 0.07$) in low FE compared with high FE mitochondria with complex I inhibition (Figure 4B). With malate-pyruvate, increases in electron leak above basal levels were observed in both groups with rotenone, antimycin A, and myxothiazol inhibition, but there were no differences between groups within inhibitor (Figure 4C). With pyruvate as an energy substrate, H$_2$O$_2$ production was increased above basal values in both groups with rotenone and antimycin A inhibition but not with myxothiazol. The H$_2$O$_2$ values were also higher ($P < 0.06$) in low FE compared with high FE mitochondria after complex I inhibition with rotenone (Figure 4D). We also observed that the magnitude of H$_2$O$_2$ production was 10-fold lower in mitochondria provided pyruvate (Figure 4D) compared with malate (Figure 4B), with the pyruvate-malate combination (Figure 4C) attenuating electron leak.

### DISCUSSION

Cellular energy (ATP) generated in mitochondria via oxidative phosphorylation fuels nutrient absorption and transport in the small intestine. A considerable amount of energy is used by the gut in carrying out its function and for the maintenance of this tissue because the intestinal epithelium is continuously renewed. New cells are produced in the crypts, migrate to villus tip, and are sloughed off in 3 to 5 d. McBride and Kelly (1990) estimated that 11 to 18% of the whole energy expenditure in ruminants is used by the gut, and the majority of this energy is spent for Na$^+$/K$^+$-ATPase activity (6 to 12%) and protein synthesis (4.0 to 4.6%). For this reason, the absorptive capacity of the small intestine is directly influenced by the availability of ATP to fuel Na$^+$/K$^+$-ATPase and to renew epithelial cells. The gastrointestinal tract consumes a large quantity of energy and uses a large proportion of the body’s oxygen supply. The contribution of the gastrointestinal tract to the whole body oxygen consumption has been reported to be approximately 20% in ruminants (Webster, 1980; Huntington and McBride, 1988), 25% in pigs (Yen et al., 1989), and 6 to 8% in broiler breeder hens (Spratt et al., 1990). Hence, inefficiencies in mitochondrial function such as increased mitochondrial ROS production may limit the amount of nutrients absorbed and may reduce the efficiency of converting feed into demand tissues or eviscerated body mass.

Unlike reports demonstrating the impact of breed (e.g., Mukherjee et al., 1970; Dziewiecki and Kolata, 1976; Brown et al., 1986) or diet (e.g., Renner et al., 1979; Toyomizu et al., 1992a, b) on mitochondrial function, experiments conducted by Bottje et al. (2002), by Lutz and Stahly (2003), and in this study used animals from the same genetic line and provided the same diet. Similar to previous findings (Bottje et al., 2002), the high FE birds in these experiments showed higher body weight gain at 7 wk due to greater FE. Evaluation of duodenum weight, length, diameter, area, or mucosa weight did not show differences between the low and high FE broilers. Thus, physical attributes of the intestines could not account for differences in mitochondrial function or H$_2$O$_2$ production observed in this study.

Mitochondrial function in isolated duodenal mitochondria was studied using established indices of mitochondrial function: RCR, for respiratory chain coupling and ADP.O ratio for oxidative phosphorylation (Estabrook, 1967). The RCR and ADP.O ratio was improved in both groups following a sequential addition of ADP in duodenal mitochondria provided with succinate, malate, pyruvate, or malate-pyruvate as energy substrates.

#### TABLE 4. Mitochondrial function in duodenal mitochondria from broilers with high and low feed efficiency (FE)$^1$

<table>
<thead>
<tr>
<th>Variable</th>
<th>Succinate</th>
<th>Malate</th>
<th>Malate-pyruvate</th>
<th>Pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High FE</td>
<td>Low FE</td>
<td>High FE</td>
<td>Low FE</td>
</tr>
<tr>
<td>RCR 1$^*$</td>
<td>3.41 ± 0.32$^*$</td>
<td>3.40 ± 0.32$^*$</td>
<td>4.65 ± 0.47$^*$</td>
<td>4.77 ± 0.31$^*$</td>
</tr>
<tr>
<td>RCR 2</td>
<td>8.89 ± 0.26$^*$</td>
<td>6.77 ± 0.81$^*$</td>
<td>9.77 ± 0.85</td>
<td>9.1 ± 0.46</td>
</tr>
<tr>
<td>ADP.O 1$^+$</td>
<td>1.19 ± 0.07$^*$</td>
<td>1.25 ± 0.13$^+$</td>
<td>2.35 ± 0.05$^+$</td>
<td>2.67 ± 0.15$^+$</td>
</tr>
<tr>
<td>ADP.O 2</td>
<td>1.72 ± 0.19$^+$</td>
<td>1.57 ± 0.10$^+$</td>
<td>2.83 ± 0.08$^+$</td>
<td>2.97 ± 0.03$^+$</td>
</tr>
</tbody>
</table>

$^*$Means within rows are significantly different between FE group within an energy substrate ($P < 0.05$).

$^+$For details, see Materials and Methods. Values represent the mean ± SEM for high (n = 6) and low (n = 7) FE broilers. Functional measurements include the respiratory control ratio (RCR) and the adenosine diphosphate (ADP):O ratio, after first (1) and second (2) addition of ADP in duodenal mitochondria provided with succinate, malate, pyruvate, or malate-pyruvate as energy substrates.

$^2$The ADP:O 2 were significantly higher than ADP.O 1 within each column ($P < 0.05, \; \ddagger P < 0.08$).

$^*$The RCR 1 values were significantly lower than RCR 2 values within each column ($P < 0.005$).
low FE mitochondria exhibited a lower RCR after the second addition of ADP due to a higher state 4 (resting) respiration rate, indicating that electron transport was less tightly coupled in the low FE than in high FE duodenal mitochondria. Lower RCR values as a result of increased state 4 respiration rate was also observed in muscle and liver mitochondria (Davies et al., 1982) and heart mitochondria (Ji and Mitchell, 1994) after exercise, which suggested possible inner mitochondrial membrane leakage (Ji, 1999). During exercise demand for oxygen consumption greatly increases. As the gut also consumes an enormous amount of oxygen and as there was greater electron leak in low FE mitochondria provided succinate (Figure 4A), there may be similar mechanisms in mitochondrial membrane leakage during exhaustive exercise and in highly metabolically active mitochondria found in intestinal tissue. With NADH-linked substrates, the RCR values were not different between groups either after the first or second addition of ADP. This result is in contrast to the findings of Bottje et al. (2002) who reported that RCR 1 was lower in low FE breast muscle mitochondria. Results of the functional studies suggest that low FE duodenal mitochondria exhibit inefficiency in respiratory chain coupling associated with complex II.

Despite the higher RCR 2 value observed in high FE mitochondria oxidizing succinate, there were no differences in the ADP:O values between the high and low FE duodenal mitochondria. The ADP:O ratio after the second
addition of ADP, however, was significantly higher in low FE mitochondria with malate, malate-pyruvate, and pyruvate as energy sources. Thus, low FE duodenal mitochondria exhibited an ability to carry out oxidative phosphorylation that was equal or superior to that of high FE mitochondria depending upon the energy substrate. We initially hypothesized that the higher ADP:O ratio in low FE mitochondria after the second addition of ADP with NADH-linked substrates might be a compensatory mechanism for a respiratory chain defect. Furthermore, the mechanism responsible for the improvement of RCR and ADP:O ratio in low and high FE mitochondria after the sequential ADP addition is also not clear at this time. But as differences in low and high FE mitochondrial function were observed only after the second addition of ADP, it appears that the 2 groups respond differently during repeated demand for energy. More experiments will be conducted to help us understand the basis of these observations.

Generation of ROS is associated with the normal functioning of mitochondria. Impairment of the mitochondrial respiratory chain leading to increased electron leak (Wei et al., 2001) and increased ROS level pose a serious threat to the cellular antioxidant defense system and increase the susceptibility of various cellular components to oxidative damage (Kristal et al., 1997; Ji, 1999). Similar to previous results in muscle (Botte et al., 2002), basal H$_2$O$_2$ production was greater in low than in high FE duodenal mitochondria with either NADH- or FADH$_2$-linked substrate (see Figure 4). This may in turn predispose the low FE mitochondria to greater oxidative stress. With succinate, inhibiting electron transfer from complex II to ubiquinone with TTFA raised H$_2$O$_2$ levels above basal values in low FE mitochondria, and these values were higher compared with those of high FE mitochondria. Low FE mitochondria also exhibited higher H$_2$O$_2$ levels compared with high FE mitochondria with malonate, another complex II inhibitor, but inhibited values were not elevated above basal levels. A possible explanation for this difference is that, as an analog of succinate, malonate is a strong competitive inhibitor of succinate dehydrogenase that blocks the tricarboxylic acid cycle (Koeppen and Riley, 1987; Lehninger et al., 1993) and, therefore, would not be expected to raise H$_2$O$_2$ values above basal levels. The results of site-specific defects evaluation with succinate suggest that the lower RCR and higher state 4 respiration after the second ADP addition observed in low FE mitochondria may be due to the higher electron leak within complex II of the electron transport chain. Earlier studies (Iqbal et al., 2001; Botte et al., 2002; Tang et al., 2002) also suggested that lower coupling might be associated with higher electron leak.

On the other hand, when complex I or NADH-linked substrates were used as an energy source, both the high and low FE mitochondria exhibited increased H$_2$O$_2$ production after inhibition of electron transport at complex I with rotenone and within center $i$ and center $o$ of complex III with antimycin A and myxothiazol, respectively. These results indicated that site-specific defects at complexes I and III in the electron transport chain appear to occur in both the high and low FE mitochondria. These results differ from findings of Botte et al. (2002) in high FE muscle mitochondria that did not show increased H$_2$O$_2$ production after inhibition of electron transport at complexes I and III. This difference in terms of ROS production between muscle and intestine may be due to the physiological function of these tissues. Several authors have suggested that ROS can induce apoptosis in many different cell systems (e.g., Simon et al., 2000). A similar ROS-mediated mechanism may be instrumental in mediating rapid turnover of intestinal epithelial cells, which would not be desirable in muscle tissue. The magnitude of H$_2$O$_2$ production at complex I with malate or pyruvate and at complex II with succinate, however, was significantly greater in the low FE compared with high FE duodenal mitochondria, which indicated the possibility of greater site-specific defects in low FE duodenal mitochondria. Attenuation of H$_2$O$_2$ production at complex I was observed when a combination of malate and pyruvate was used as energy substrate. The higher ADP:O ratio after the second addition of ADP observed in low FE mitochondria with malate or pyruvate but not with malate-pyruvate combination may indeed be a compensatory mechanism in low FE mitochondria as a result of higher electron leak or H$_2$O$_2$ production with these substrates.

The well-known sites of O$_2$$^-$ and H$_2$O$_2$ generation are complexes I and III of the electron transport chain (Chance et al., 1979; Turrens and Boveris, 1980; Nohl et al., 1996). Site-specific defects have been observed at complexes I and III in lung, heart, and breast muscle mitochondria in broilers with pulmonary hypertension syndrome (Iqbal et al., 2001; Ji, 1999). As such, this may reflect the metabolic function of the tissue. Conversely, Cawthon et al. (2001) reported site-specific defects at complex II in liver mitochondria from PHS broilers. In the current study, site-specific defects in electron transport were observed at complexes I, II and/or III (depending on substrate) in the high and low FE duodenal mitochondria. Combined, these results imply that the site of electron leak in the mitochondrial respiratory chain may be tissue dependent as suggested earlier by Kwong and Sohal (1998) and may reflect the metabolic function of the tissue.

The H$_2$O$_2$ data also suggest that electron leak in duodenal mitochondria may depend on the energy substrate. In addition, H$_2$O$_2$ values with pyruvate were 10-fold lower compared with succinate, malate, or malate-pyruvate in both FE groups. Because substrates used in this study were chosen based on acceptable RCR values during test runs, the lower magnitude of H$_2$O$_2$ with pyruvate was unexpected. However, in studies on lymphoid cell lines (Miwa et al., 2000) and in inflammatory joint diseases (Herz et al., 1997), it has been reported that pyruvate can directly scavenge H$_2$O$_2$ and protect cells from ROS-mediated oxidative damage. In plant mitochondria, pyruvate can also inhibit H$_2$O$_2$ formation by activating the alternative oxidase (Braidot et al., 1999). Thus, lower levels of H$_2$O$_2$ formation with pyruvate observed in this
study may be due to its action as an antioxidant or through its ability to stimulate other antioxidant defenses in the cell.

In summary, the results of these experiments demonstrated that mitochondrial electron transport chain coupling was higher in high FE mitochondria following repeated demand for energy with FADH₂ but not with NADH-linked substrates. However, with NADH-linked substrates low FE duodenum mitochondria exhibited an ability to synthesize ATP in vitro that was superior to substrates low FE duodenum mitochondria. This research was presented in both low and high FE duodenal mitochondria at complexes I and III. The magnitude of site-specific defect at complexes I and II, however, was significantly higher in the low FE mitochondria, suggesting that increased electron leak at these sites was associated with low feed efficient broilers. The findings in this study provide insight into understanding the relationship between FE and mitochondrial function in duodenal tissue. A better understanding of cellular and biochemical mechanisms associated with FE could help in developing tools for aiding selection programs in identifying breeding replacement stock with superior FE.

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